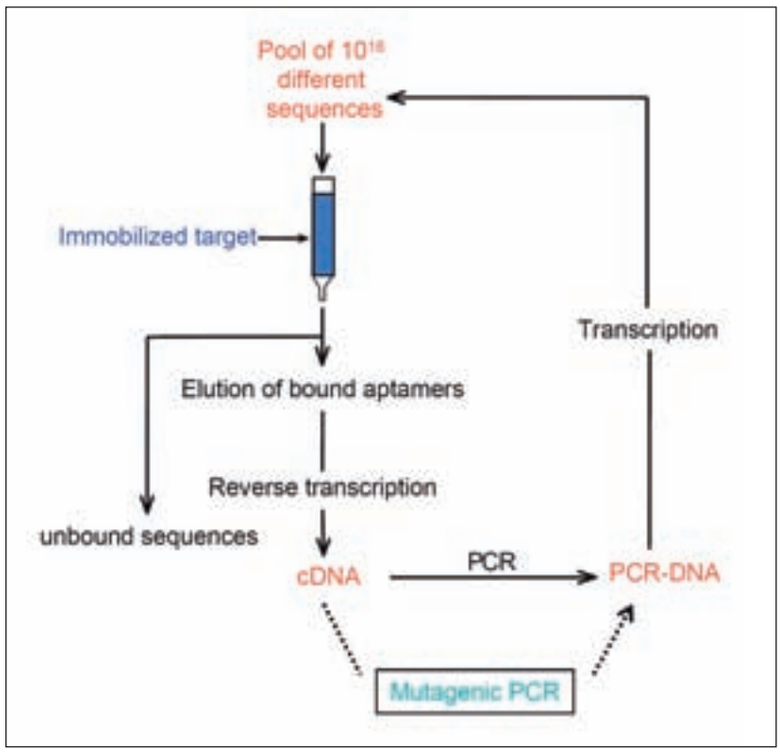
The power of APTAMERS in the drug discovery process

Methods of combinatorial biochemistry allow the identification of nucleic acid sequences, or aptamers, which can bind their target molecules with high affinity and specificity and are able to efficiently inhibit their biological function. Macugen, the first aptamer-based drug for the treatment of age-related macular degeneration has been approved and is now on the market. Aptamers thus represent a promising class of novel therapeutics. However, aptamer technology has opened up many more different applications. Recent work has established aptamers as a powerful tool for the identification of low molecular weight lead compounds.

By Professor Michael Famulok

Aptamers are short single-stranded nucleic acids that can bind with high affinity to a wide variety of protein targets. The designation 'aptamer' is a linguistic hybrid composed of latin word 'aptus', for 'fitting' and the greek suffix 'meros', for 'a part'. An aptamer is thus a 'fitting part'. The first aptamers were described in 1990 by Larry Gold[1] and independently by Jack Szostak[2]. Since then, hundreds of aptamers have been selected that recognise a plethora of targets such as small molecules, peptides, proteins and even living cells or viruses[3]. The process by which aptamers can be isolated is straightforward, and is often referred to as '*in vitro* selection' or 'SELEX', an acronym for 'systematic evolution of ligands by exponential enrichment'. Here, complex libraries of randomised nucleic acid sequences are systematically enriched in iterative cycles of selection and amplification, consisting of the steps (a) incubation with the target molecule, (b) separation of bound from unbound sequences, (c) amplification of bound nucleic acids (**Scheme 1**). Depending on the nature of the target, the number of sequences present in the initial library that fulfill a selection criterion, the complexity of the library, and the stringency of the selection process, the number of selection cycles required for the enrichment of active sequences can vary from just a few up to 20. The nucleic acid library used for the selection can be structured modularly, depending on the desired outcome. In a standard library, a randomised sequence that can vary considerably in length is flanked by known sequences of approximately 20 nucleotides that serve as primer binding sites that allow reverse transcription (in case of RNA libraries) and amplification of selected sequences by the polymerase chain reaction (PCR). The ease of amplification of minute amounts of active sequences allows libraries of nucleic acids to be of extraordinary complexity. It is possible to search through no less than 10^{16} different molecules in a single selection experiment. No other combinatorial process can sift through such huge numbers of



Scheme 1
In vitro selection of aptamers

different molecules at once. Impressively, this number even exceeds by far the number of antibodies that can be generated by the immune system of higher organisms.

So far, SELEX experiments are mostly carried out *in vitro* which bears the inherent advantage compared to, for example, the standard generation of antibodies, that target molecules can be addressed that are toxic or would be lethal to an organism, or that are difficult to address by alternative methods for different reasons. The selection process *in vitro* also allows precise control about the course of the selection and thus, some manipulation of the properties of the resulting aptamers is possible. In addition, selected sequences that do not quite come up to the researcher's expectations, can be further optimised in subsequent *in vitro* selections that involve further mutagenising selected sequences and an increase of the selection stringency. In this way, the principles of Darwinian evolution can be applied in a highly straightforward manner to enhance the quality of an aptamer (**Scheme 1**).

Aptamers have been used in multifaceted applications ranging from diagnostics to therapeutics; from employments as research tools in molecular biology and target validation to nanotechnology. New fields of application for aptamer technology in basic research are constantly emerging, for example in Chemical Biology, Chemical Genetics, Medicine and Biotechnology. Although aptamers are similar to antibodies in their universalism as research tools, the fact that they can be accessed synthetically by standard oligonucleotide synthesis is advantageous in several respects. For example, it is straightforward to equip an aptamer synthetically with additional chemical functionalities such as fluorescent groups, affinity labels, nanoparticles, reactive groups, crosslinkers and other modifications.

Therapeutic aptamers

In the year 2004, 14 years after the pioneering description of the *in vitro* selection of aptamers, the first aptamer was approved as a drug by the Food and Drug Administration (FDA)[4]. This aptamer is known under the trade-name Macugen® (Pegaptanib sodium injection) and is used for the treatment of age-related macular degeneration (AMD). Macugen is a highly modified RNA aptamer which binds with high affinity to vascular endothelial growth factor (VEGF) and interferes with its interaction with the VEGF-receptor. In patients suffering from AMD, the choroidal blood vessels and the retinal pigment epithelium exhibit increased expression levels of VEGF. Thus, the inhibition of VEGF blocks neovascularisation in the eye in a therapy which involves intravitreal injection of 0.3mg aptamer once every six weeks.

Crucial for the successful conversion of the original *in vitro* selected anti-VEGF aptamer was its stabilisation and equipment with a variety of chemical modifications. RNA molecules usually are highly prone to degradation by nucleases which are present in virtually any body fluid. Therefore, an aptamer used for therapy must be stabilised towards hydrolysis by nucleases. There are several ways for stabilising an aptamer sequence. Simply by exchanging the 2'-OH group of pyrimidine residues in an RNA sequence for 2'-fluoro- or 2'-amino residues it is possible to increase its stability towards nucleases considerably. Advantageously, these modified pyrimidine nucleotide triphosphates can serve as substrates of RNA-replicating enzymes used in the *in vitro* selection process, in particular RNA polymerase and reverse transcriptase. This compatibility with the general aptamer selection protocol allows the *de novo* isolation of aptamer sequences containing these modifications in their pyrimidine residues which lends them enhanced nuclease resistance *per se*. Once identified as a functional sequence, additional modifications can be introduced synthetically, leading to a

further increase of stability. The anti-VEGF aptamer was originally isolated in this way in the form of a 2'-deoxy-2'-fluoropyrimidine RNA molecule. Except for the adenosine residues in positions 4 and 5, all other 2'-OH groups of the purine residues could be substituted by 2'-methoxy groups which resulted in dramatically increased stability towards nuclease degradation without loss of its VEGF binding affinity (**Figure 1**).

Another problem that needed to be overcome was that, in general, the half-life of nucleic acids after intravenous injection is short due to rapid clearance via the kidneys. However, by modifying the 5'-end with polyethylene glycol moieties (PEG linker) it is possible to increase the blood residence time significantly. In case of Pegaptanib, a 40kDa PEG linker was attached to the 5'-terminus of the aptamer which led to a half-life of 10 ± 4 days in the eye. In addition, to protect the sequence against degradation by exonucleases, the 3'-end was modified with an unusual 3'-3'-phosphate linker by attaching a 3'-3'-deoxythymidine cap moiety (**Figure 1**). Despite its high stability, the aptamer is metabolised by nucleases within several weeks and is excreted via the urinal tract.

There are other aptamers currently in clinical and pre-clinical trials. The various fields of therapy comprise acute myeloid leukemia (AML), cardiovascular diseases, metabolic disorders, and anticoagulants. Particularly in the latter field, aptamer technology appears promising, for example during coronary artery bypass graft (CABG) surgery. Aptamer-based anticoagulants have the big advantage compared to standard drugs that they can be neutralised extremely quickly, simply by administering another stabilised nucleic acid sequence that is complementary to the aptamer. This antisense sequence efficiently hybridises to the functional aptamer and immediately transforms its functional three-dimensional fold into a double helix that no longer can bind the target molecule. In this way, the antisense sequence acts as an antidote that neutralises the antagonising effect of the aptamer. This is essential to avoid unwanted side-effects such as post-operative bleeding, and is also important in interventions that involve the temporal extracorporeal repositioning of the blood circuit. It already could be demonstrated in animal models that the anticoagulating action of an aptamer directed against the blood coagulation factor IXa was rapidly and effectively inactivated after administration of an antisense molecule antidote[5]. In another application, variants of an aptameric inhibitor of human α-thrombin were covalently attached to an antisense strand. However, the ability to base-pair with the aptamer was blocked temporarily by the strategic positioning of a photolabile protecting group. This protecting group could be quantitatively removed by irradiation at λ = 366nm which resulted in activating the antidote activity of the antisense strand. In this way, an anticoagulant was designed that inherently contained anticoagulatory as well as antidote activity and thus antagonises itself after irradiation. Depending on the status of irradiation, either one or the other function was active[6].

These examples illustrate the therapeutic potential of aptamers and show that aptamer-based therapeutics allow controlling the activity of a drug in a way that is difficult to achieve with drugs that are based on non-nucleic acid based biologicals.

Aptamers and ribozymes as tools for drug discovery

Without exception, the target proteins for which aptamers with potential therapeutic applications have been developed or are currently under development are extracellular. The reason obviously lies in the nucleic acid nature of this type of therapeutic:

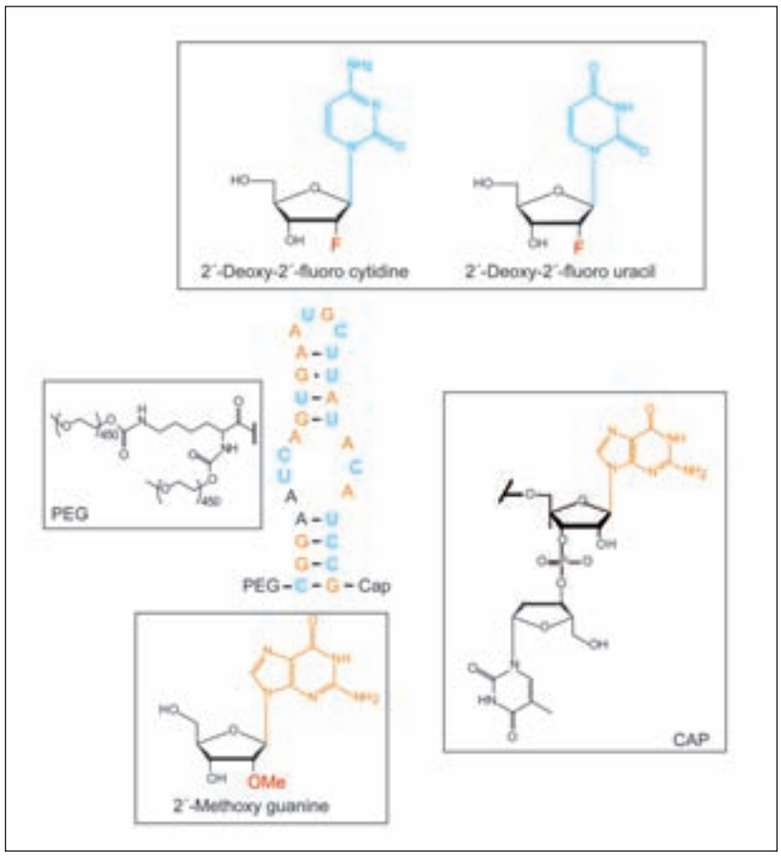


Figure 1
Pegaptanib for the treatment of AMD. The anti-VEGF aptamer Pegaptanib contains a number of modifications that lead to stabilisation towards ribonucleases and exonucleases and to an increase of its half-life in blood plasma

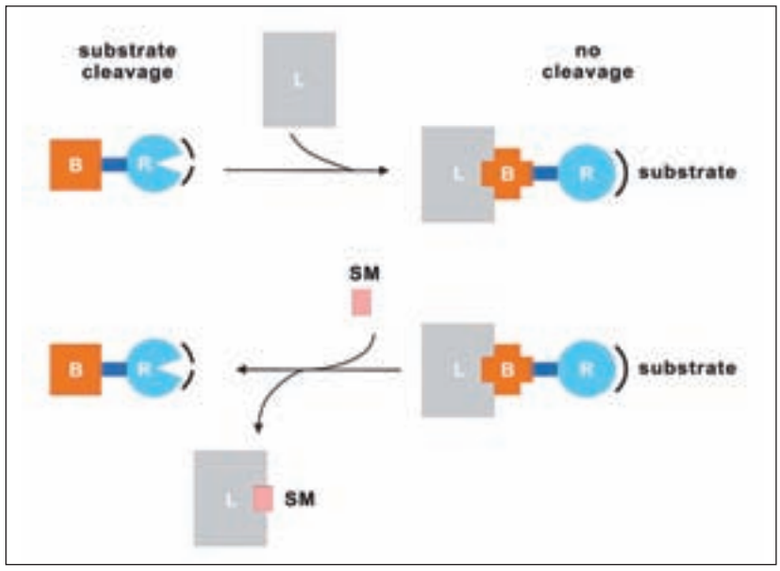


Figure 2
Schematic of an allosteric ribozyme and reporter ribozyme. The allosteric ribozyme is illustrated by the combination of aptamer domain (B; orange) and the ribozyme domain (R; blue). In the upper panel, the addition of the protein ligand (L; gray) induces a conformational change in the ribozyme, resulting in the inhibition of substrate (black line) cleavage. The lower panel represents a reporter ribozyme used in a competitive assay. The small molecule (SM; pink), which interferes with the interaction of ligand-ribozyme, restores the active conformation of ribozyme. Then, substrate cleavage can be observed in real-time

in order to target an intracellular protein, the aptamer would have to cross the plasma membrane, a requirement that is far from trivial to solve for a highly negatively charged molecule. It is therefore not surprising that until now no aptamer targeting an intracellular protein has been brought close to entering clinical trials. However, several examples have shown that aptamer technology can serve as a link between target validation and the identification of small drug-like molecules as lead compounds.

Imagine, for example, an intracellular target protein for which no inhibitory molecule is available that could be applied to answer questions like drugability of the target, the physiological consequences of its inhibition, phenotypic changes, etc. In such cases it would be highly desirable to have rapid access to a specific inhibitor that could be applied inside cells to answer these questions, independent of whether the inhibitor can be turned into a drug, or not. The criterion of rapidity certainly is fulfilled by aptamer technology. Hundreds of examples have established that an inhibitory aptamer can be generated for virtually any protein target within short time. This is mainly due to the fact that as much as 10^{16} different sequences can be generated at once and one single active sequence can, in principle, be identified from this complex mix. Therefore, an aptamer library has much more variability than a standard drug library with the consequence that the probability of success is dramatically increased. A functional aptamer can then be used in preliminary 'validation studies' to answer the questions listed above in cell-culture studies in which the aptamer can be investigated in an intracellular context by applying transfection techniques. A plethora of methods for bringing a nucleic acid into a cultured cell has been developed in the context of nucleic acids that downregulate the expression of mRNAs which they target, such as short interfering RNAs (siRNAs), antisense oligonucleotides, or ribozymes. The same methodology also works for aptamers with the difference that the aptamer directly targets the protein rather than the expression of a gene. If the application of the aptamer leads to promising results the next step then would be to find a small molecule with similar inhibitory potential to that of the original aptamer, which would allow, for example, the shift from cell culture studies into animal models to characterise the endogenous target protein in the context of a living organism.

Several examples have shown that aptamers can serve as versatile reporters in screening assays in which compound libraries are searched for small molecules that are able to interfere with the binding of an aptamer to its ligand protein. This is particularly useful for proteins for which it is difficult to establish a functional assay.

There are several ways of setting up an aptamer-based screening assay. The initial examples were based on allosteric ribozymes, in which the activity of a catalytic RNA, or ribozyme, is controlled by a regulatory aptamer sequence in an allosteric mechanism in which conformational changes that the aptamer undergoes when binding to its target are relayed to the catalytic centre of the ribozyme.

In one application, we have fused an aptamer that binds to HIV-1 reverse transcriptase (RT) to a ribozyme[7]. The presence of RT induces the formation of a different structure of the aptameric portion. Thereby, the binding of RT to the aptamer prevents the ribozyme from cleaving a small oligonucleotide substrate RNA, labelled with a fluorescent dye and a fluorescence quencher. Due to fluorescence resonance energy transfer (or 'FRET'), fluorescence of the uncleaved substrate is quenched. In the absence of RT the reporter ribozyme remains active, and substrate cleavage can be followed in real-time by an increase in fluorescence. This effect was highly specific for HIV-1 RT. The homolog RT of HIV-2 was not detected. Thus, the reporter-ribozyme served as a specific biosensor that signalled the presence of HIV-1 RT. In this sense, reporter-ribozymes supplement currently used antibody-based techniques like ELISA while being considerably more straightforward due to real-time readout in solution and other advantages. When the protein is displaced from the reporter-ribozyme by interacting with a small

drug-like molecule, the reporter-ribozyme can again cleave the substrate, resulting in a fluorescence signal (**Figure 2**).

Using this assay, we identified a small molecule inhibitor of HIV-1 RT that inhibited the DNA-dependent, but not RNA-dependent primer elongation activity of the enzyme[8]. This small molecule reduced the replication of wild-type and a multidrug-resistant strain in a mechanism in which the compound competed with primer/template complex binding to HIV-1 RT. An analysis of available crystallographic data of HIV-1 and HIV-2 RTs, constrained by the characteristics of drug resistance mutations and the inhibitory profile of the compound suggested a region in these proteins as a new drugable interface that can be targeted by a small molecule.

In another application, Srinivasan et al used an ADP responsive allosteric ribozyme ('RiboReporter') to monitor the enzymatic reaction of a protein kinase[9]. Thereby, new protein kinase inhibitors or compounds that modulate the activity of any enzyme which is involved in ADP metabolism can, in principle, be identified. The authors demonstrated that RiboReporter was able to rediscover a known protein kinase inhibitor through screening.

Aptamers can also be used directly for screening. The chemical synthesis of aptamers allows their labelling with reporter molecules that are compatible with many high-throughput screening assay formats such as fluorescence resonance energy transfer (FRET), fluorescence polarisation and enhanced chemiluminescence.

We have applied this technology to find a small molecule inhibitor for a class of proteins, called cytohesins that regulate the activation of G proteins by catalysing the exchange of GDP for GTP[10]. Cytohesins are a class of guanine nucleotide exchange factors (GEFs) that are insensitive to Brefeldin A (BFA) the only known small molecule inhibitor of GEFs. We screened a small-molecule library by fluorescence polarisation for a compound that could displace a fluorescein-labelled inhibiting RNA aptamer from the cytohesin GEF domain and identified SecinH3, a small molecule that bound cytohesin and inhibited its GEF activity. By feeding SecinH3 to flies[11] and mice[10] it could be established that cytohesins play an essential role in the insulin signalling cascade.

These examples show that compounds identified in aptamer displacement screens or allosteric ribozyme-based assays exhibit similar properties as their parent aptamers. They bind to the same domains in their target proteins and can inhibit their biological function. In contrast to the aptamer itself, the small molecule enables a simple application in animal models and might thus serve as a starting point for drug-development. The versatile application potential of aptamers can thus be efficiently combined with the identification of small molecule lead compounds.

Conclusion

Aptamers are potent protein inhibitors and can contribute like hardly any other compound class to the drug discovery process. With Macugen, the first therapeutic aptamer is available for treating AMD. Besides their direct use as therapeutics, aptamers can also be applied for validating pharmacologically relevant proteins as well as for the identification of low molecular weight protein inhibitors. The aptamer technology thus represents a technology platform whose versatile fields of applications range from the functional characterisation of biomolecules to drug development. DDW

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